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Candida Species Adhesion to Oral Epithelium: Factors Involved and Experimental Methodology Used

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Due to the increasing prevalence and emergence of Non-*Candida albicans Candida* (NCAC) species, especially in immunosupressed patients, it is becoming urgent to deepen the current knowledge about virulence factors of these species. Adhesion of cells to epithelium is considered one of the major virulence factors of *Candida* species. However, relatively little is known concerning the adhesion mechanisms of NCAC species to epithelium, as well as about the factors affecting the adhesion process. This review focuses both the mechanisms that regulate the adhesion interactions and the factors involved and the description of the experimental methodology that has been used to perform the adhesion assays.

Keywords Adhesion; *Candida*; Species; Oral epithelium

INTRODUCTION

Oral Candidiasis is one of the most common pathologies encountered in patients with HIV infection, developing in over 80% of these individuals at some time during their illness. In non-immunocompromised patients, this infection can often be eradicated with a short course of topical or oral treatment with an azole compound. However, because of the profound and sustained immunosuppression in patients with AIDS, persistent or recurrent oral infection with *Candida* species is common and long courses of antifungal treatment are required if remission from infection is to be achieved and sustained (Jacobs and Nall 1997). According to Odds (1987), most people usually carry a single strain of *Candida* at different body sites for a long time, for instances *Candida dubliniensis* can be found in the oropharynx or in the upper respiratory tract (Sullivan et al. 2005) while *Candida albicans* is most prevalent in the palate, tongue and gingiva. However, it has been shown that a few individuals may harbour more than one strain or species of *Candida* at the same time, and that in hospitalized and immunocompromised patients this occurs more commonly (McCullough et al. 1996).

Although *Candida* species are commensal, the median reported prevalence of oral yeast carriage in the general population is 34% while in hospitalized patients it rises to 55% (MacFarlane 1990). The attributable mortality of *Candida* infections is as high as 38% (Wey et al. 1988).

Whether *Candida* simply remains as a commensal or proliferates, invading tissues and producing Candidiasis is determined by changes in the environment of the host. The commensal relationship is dependent on the maintenance of host tissue integrity with normal microbial flora as well as on an intact immune system. As long as these host conditions are maintained, mucosal Candidiasis is not observed clinically. However, a breakdown in anatomic integrity or a change in the resident microbial flora can lead to environmental conditions that are favourable for the growth of *Candida spp.* with potential for host invasion by the fungus. Such environmental conditions in conjunction with an imbalance of host cytokine response can lead to increased tissue colonization and fungal overload resulting in mucosal Candidiasis (Calderone 2002a). The infections produced range from the superficial to the systemic. The latter type is mainly observed in individuals with immunological deficiencies and represents an important clinical problem (Pla et al. 1996).

Among the several *Candida* species, *Candida albicans* is by far the most studied. Although, other *Candida* species are emerging pathogens, namely: *C. parapsilosis*, *C. tropicalis*, *C. guilliermondi*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. kefir,* and *C. dubliniensis*, which are now generally referred to as non-*Candida albicans Candida* (NCAC) species. The prevalence of these species has been changing along the years. In the 1980s, according to the studies made by Kiehn et al. (1980), *C. albicans* constituted 68% of isolates from sites other than blood in cancer patients, while *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei* accounted for 12.3, 10.3, 3.0, and 1.5% of isolates, respectively. In representative studies of fungemia in immunocompromised hosts, diabetics, neonates and surgical patients *C. albicans* again accounted for 60–80% of the isolates, while other *Candida* species were identified less than 20% of the time (Butler and Baker 1988). In the same decade it was also

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reported that 70–78% of the isolates were *Candida albicans* (Finlay 1986). Nevertheless, studies from the 1990s reported only 56–57% of the isolates as *C. albicans* (Jobbins et al. 1992), revealing the emergence of NCAC species. Davies et al. (2002), assessed the presence of *Candida* species in oral rinses from patients with cancer and found 46% of *C. albicans*, 18% of *Candida glabrata*, 5% of *Candida dubliniensis,* and less than 5% of other non-*Candida albicans Candida* species. This value of approximately 50% of *Candida albicans* prevalence was also confirmed by Borst et al. (2003). More recently, 40% of the fungemia cases reported by Bassetti et al. (2006) were due to *Candida albicans*.

The most similar NCAC species to *Candida albicans* is *Candida dubliniensis* presenting a very analogous genotype and phenotype, which led to their misidentification for years. The most conclusive evidence demonstrating that *Candida dubliniensis* was distinct from other *Candida* species was generated by phylogenetic analysis of nucleotide sequences encoding de V3 region of the larger rRNA subunit gene (Sullivan et al. 1997). This species was only identified in 1995 (Sullivan et al. 1995) and it was primarily associated with oral carriage and infection in HIV-infected and AIDS patients. The prevalence of *Candida dubliniensis* in these patients indicates that due to severe immunodeficiency this organism can emerge as an opportunistic pathogen, probably from the patients' own flora. In this environment, the ability of *C. dubliniensis* to adhere strongly to oral epithelial cells may provide it with a competitive advantage over other, less adherent, NCAC species (Gilfillan et al. 1998; McCullough et al. 1995). There are some *Candida dubliniensis* isolates that are resistant to fluconazole and this ability may confer a further selective advantage to *C. dubliniensis* in HIV-infected individuals receiving long-term maintenance therapy for suppression of oral Candidiasis. However, this antifungal susceptibility is not as strong as it is in *C. krusei* and *C. glabrata* (Pfaller et al. 2002). The increase in *Candida glabrata* systemic infections is a subject of considerable concern due to the tendency of this species to rapidly develop resistance to azole antifungal agents and due to the high mortality rate associated with *C. glabrata* fungemia (Fidel et al. 1999). Although this species is second only to *C. albicans* as a cause of systemic Candidiasis, it is a pathogen of low virulence and infection is usually associated with severely debilitated patients (Komshian et al. 1989). Infections caused by *Candida krusei* are prevalent in patients receiving prophylactic fluconazole therapy (Rex JH et al. 2000). Some studies (Chavanet et al. 1994) described the replacement of *C. albicans* with *C. krusei* in the oral cavities of HIV-infected patients following azole therapy. Although *C. krusei* is inherently resistant to fluconazole, some authors (Berrouane et al. 1996; Kao et al. 1999) have also noticed reduced susceptibility to other antifungal drugs among isolates of *C. krusei*, leading to the fact that *C. krusei* is a multidrug-resistant pathogen. The other two *Candida* species better known are *C. parapsilosis* and *C. tropicalis*: the former is the second most frequently recovered from blood culture and the latter the second most virulent. *Candida parapsilosis* is commonly recovered from human skin and can adhere strongly to the surfaces of intravascular catheters and prosthetic devices (De Bernardis et al. 1999; Levin et al. 1998). In contrast to the other NCAC species already described here, *C. parapsilosis* is generally susceptible to all of the major antifungal drugs, as well as *C. tropicalis*. The latter has been reported as the NCAC species most commonly recovered from blood culture in patients with cancer (Komshian et al. 1989; Wingard 1995). However, it has been shown that during the recent shifts in the epidemiology of Candidiasis, *C. tropicalis* has now been superseded by *C. glabrata* and *C. parapsilosis* in overall importance (Pfaller et al. 2000; Berrouane et al. 1999).

In vitro studies have demonstrated that NCAC species generally are less adherent to buccal epithelial and vascular endothelial cells, secreting less proteinases than *C. albicans*, which may account for their reduced virulence (Hube 1996; King et al. 1980). *C. albicans* is undoubtedly the most virulent *Candida* species, followed by *C. tropicalis* (Ghannoum and Abu-Elteen 1991). This virulence of *C. tropicalis* may be due to its greater ability to adhere to epithelial cells and its ability to secrete moderate amounts of proteinase relatively to the other NCAC species (King et al. 1980; Zaugg et al. 2001). *C. parapsilosis* seems to be less virulent (De Bernardis et al. 1999) followed by the other NCAC species.

In order to proliferate in the oral cavity, yeast cells must adhere to the oral surfaces otherwise they are washed out by the salivary flows. So, one of the most important factors of virulence of *Candida* species is their ability to adhere using a variety of mechanisms, permitting the yeast to anchor at a site and the process of tissue colonization to commence (Cotter and Kavanagh 2000). After colonizing the host's mucosal surfaces, *Candida* species may then invade beneath the mucosal barrier into the vascular space, where continued replication causes hematogenously disseminated disease (Hostetter 1994).

Although the importance of NCAC species is everyday more evident, there are still only few studies concerning them. For instance, in the recent major *Candida* meeting, "8th ASM meeting on *Candida* and Candidiasis, 2006," only 16% of the work presented concerned these species.

YEAST-EPITHELIAL CELL INTERACTIONS

The first observation that adherence of *C. albicans* could be important for virulence was provided by King et al. (1980).

Adherence to host tissue cannot be explained by one single specific event, because it can be ruled by a combination of specific and non-specific interactions.

Specific Interactions

The components of the organism that promote host recognition and colonization are referred to as adhesins. According to Calderone and Fonzi (2001), an adhesin can be defined as a biomolecule that promotes adhesion of microbial cells to host cells or host-cell ligands. Host cells have some components that

are recognized by *Candida* adhesins. These components can be biomolecules of different classes, namely, carbohydrates or proteins. The adhesins of *Candida albicans* are usually of polysaccharide or glycoprotein nature (Calderone and Gow 2002). Several types of yeast adhesins, cell receptors and genes, as well as secreted aspartyl proteinases (SAP) are involved in the yeast-cell specific interactions.

Epithelial Cell Factors

(i) Fibronectin. Fibronectin was one of the first molecules to be suggested as a ligand recognized by C. *albicans* adhesins (Skerl et al. 1984). Fibronectin is a plasma and interstitial tissue glycoprotein to which a number of microorganisms avidly adhere (Pendrak and Klotz 1995). This protein ranges in mass from 37 to 120 kDa. Santoni et al. (1994) found that adhesion of *C. albicans* blastospores to immobilized fibronectin containing the RGD tripeptide was inhibited by monoclonal antibodies and by the GRGDSP peptide from fibronectin. One year later the same authors (Santoni et al. 1995) described that a monoclonal antibody against placental $\alpha 5\beta 1$ and two polyclonal antibodies recognizing vertebrate fibronectin receptors also bound to NCAC species. These studies provided evidence for fibronectin receptors in *C. albicans* and *C. tropicalis* that resemble the vertebrate integrins $\alpha 5$ and $\beta 1$. In disseminated Candidiasis, the fibronectin adhesin may be responsible for the adherence of the microorganism to intravascular and vascular structures such as endothelial cells or subendothelial extracellular matrix (Pendrak and Klotz 1995). The presence of fibronectin as a target protein for epithelial attachment of *Candida tropicalis* was also confirmed by Bendel et al. (1993).

(ii) Integrins. An integrin or integrin receptor is an integral membrane protein in the plasma membrane of cells. On vertebrate cells, integrins serve a multiplicity of functions from adhesion to morphogenesis. *Candida* proteins exhibiting antigenic and functional similarities to human complement receptors 3 and 4 (CR3 and CR4) are known as integrin analogs because of the placement of CR3 and CR4 within the integrin supergene family (Hostetter 1994).

Included in the C3 ligands are C3b, C3d, and iC3b that are ligands for CR1, CR2, respectively, and CR4 (for the last two). The iC3b receptors are present on the surface of *C. albicans*, and these share homology with a subunit of the neutrophilic iC3b receptor (Lee et al. 1997) and it is proposed that a number of fungal proteins mediate *C. albicans* adherence to iC3b receptors (CR3-like). The presence of a receptor for iC3b on the surface of *C. albicans* allowed noncovalent binding of this protein, thereby suggesting that *C. albicans* was using this form of molecular mimicry to elude phagocytosis (Hostetter 1999).

The presence of a receptor for C3d on *C. albicans* and *C. stellatoidea* was initially described by Heidenreich and Dietrich (1985) and later confirmed by Edwards et al. (1986). While Heidenreich and Dietrich (1985) observed this receptor and a receptor for iC3b on both yeasts and hyphal forms, Edwards et al. (1986) only detected it on hyphal forms. Calderone et al. (1988) identified, in extracts of *C. albicans* pseudohyphae, two proteins of approximately 62 and 70 kDa that bind the C3d fragment of C3. The finding of C3 receptors, exclusively on the more pathogenic *Candida spp.* is highly suggestive of their involvement in disease processes (Calderone et al. 1988).

According to Bendel and Hostetter (1993) epithelial adhesion of *C. tropicalis* is not significantly inhibited by iC3b and iC3b-RGD peptides, however, two peptides in the group—I-RGDQD and RGDQDATMS—were more inhibitory than the remainder.

Yeast Factors

(i) Genetic regulation. It was proposed by Staib et al. (1999) that a developmentally regulated gene (HWP1), expressed in germ-tube and hyphal forms of *C. albicans*, encodes an outer cell-wall mannoprotein that interacts with epithelial cell transglutaminase, forming a non-dissociable complex. Tsuchmori et al. (2000) demonstrated that an HWP1-deficient mutant of *C. albicans* caused reduced mortality in mice, germinated less readily in the kidneys of infected mice and caused less endothelial cell damage. This confirms the role of HWP1 in adherence and virulence.

Agglutin-Like Sequence (ALS) of *Candida albicans* is a family of seven glycosylated proteins with homology to the *S. cerevisae* α-agglutinin protein that is required for cell-cell recognition during mating (Calderone and Fonzi 2001). For *C. albicans*, both ALS1p and ALS5p appear to provide an adhesive function (Gaur et al. 1999; Fu et al. 1998). ALS genes are differentially regulated in *C. albicans* by physiologically relevant conditions such as growth medium changes (ALS1) (Hoyer et al. 1995; Hoyer 2001), morphological form (ALS3/ALS8) (Hoyer et al. 1998b; Hoyer and Hecht 2000) and stage of growth (ALS4) (Hoyer et al. 1998a). Hoyer et al. (2001) observed differences in ALS gene expression by *C. albicans* and *C. dubliniensis*. In *C. albicans*, it is typical to observe one or two ALS genes concurrently expressed under a specific in vitro growth condition. By contrast, more ALS cross-hybridizing messages were observed on *C. dubliniensis* northern blots. Hoyer et al. (2001) demonstrated that ALS gene families are found in *C. dubliniensis* and *C. tropicalis*, although they are not identical to that in *C. albicans*. Studies of ALS genes in *C. dubliniensis* suggest differences in regulation of the gene family and in production of cell wall proteins.

Studies of the ALS family revealed many significant parallels to the *C. albicans* SAP family (Hoyer et al. 2001; Hube et al. 1994; Hube 1996). Both families appear to have similar numbers of genes in *C. albicans*. Genes in each family are differentially regulated by similar mechanisms and ALS and SAP are largely co-localized on the same *C. albicans* chromosomes. This co-localization trend continues in species such as *C. dubliniensis* and *C. tropicalis*. The number of ALS genes is roughly equal to the number of SAP genes in these species (Hoyer 2001).

In *C. glabrata*, adherence is mediated largely by the *EPA* (Epithelial Adhesion) family of genes, which, like HWP1 or the ALS genes encode GPI-anchored cell wall proteins. Epa1 is a

lectin that binds to N-acetyllactosamine-containing glycoconjugates (Kaur et al. 2005; Cormack et al. 1999).

The *Candida glabrata* genome encodes many *EPA*-related genes. Despite the large number of *EPA* genes, deletion of just *EPA1* reduces adherence in vitro to background levels because the other EPA genes are expressed at low levels when grown in laboratory broth (Castano et al. 2005; De Las Penas et al. 2003). The deletion of the *EPA1* gene reduces adherence by 95% (Cormack et al. 1999).

(ii) SAP. Secreted Aspartyl Proteinases (SAP) also appear to contribute to adhesion of *Candida albicans* to buccal epithelial cells (BEC) and other substrates (Watts et al. 1998; Cannon and Chaffin 1999). SAP were first described by Staib in 1965 (Staib 1965) and originally designated as CAP (*Candida* aspartyl protease). SAP 1 was described by Hube et al. (1991) and SAP2 described by Wright et al. (1992). According to White et al. (1993), there are at least three SAPs. The three proteinases isoenzymes described differ in the primary sequence, pI and pattern of expression and are products of three separate genetic loci. These differences suggested that the different proteinases may have unique roles in the interaction between *Candida* and its host. Some more SAP were identified later, including SAP4 (Miyasaki et al. 1994), SAP5, SAP6 and SAP7 (Monod et al. 1994), SAP8 (Morrison et al. 1993), and SAP9 (Monod et al. 1998).

White and Agabian (1995) studied the influence of growing conditions and cell type in the production of SAP. In their study, they defined the culture conditions that control the levels of SAP mRNAs and Sap proteins, and they indicate that both yeast/hyphal transition and phenotypic switching can determine which of the Sap isoenzymes is produced.

Some authors (Hube et al. 1997; Sanglard et al. 1997) found that in guinea pig and murine models of invasive disease, deletions in SAP1-6 attenuate virulence. Thus, it would appear that SAP1-6 is required for invasive disease. An in vitro model of human oral Candidiasis has been used to follow the temporal transcription of SAP1-8. SAP4 and SAP5 were never detected and SAP1 and SAP3 were expressed within 42 h of tissue post-infection, followed by SAP6 and SAP2 and SAP8 (Calderone and Fonzi 2001).

Genes encoding aspartyl proteinase have been cloned in *C. albicans* and *C. tropicalis* (Hube et al. 2006; Togni et al. 1991; Wright et al. 1992). Borg and Ruchel (1988) found that evidence implicating a role for aspartyl proteinase includes the demonstration of the proteinase in both blastospores and invading germ tubes of *C. albicans* and *C. tropicalis* but not in *C. parapsilosis*.

Non-Specific Interactions

Besides specific interactions, there are other types of interactions, named non-specific, which can rule the adhesion phenomenon. These interactions are directly related to cell surface properties, and are mediated by hydrophobic and electrostatic forces.

Some studies in bacteria lead to the finding that hydrophobic interactions are believed to contribute to adherence by maintaining the fidelity of the adhesin-receptors bonds (Beachey 1981; Rosenberg and Kjelleberg 1986). In relation to yeast *Candida* cells, Rotrosen et al. (1986) and Hazen (1989), described that cell surface hydrophobicity (which increases at lower growth temperature) contributes to, adherence to BEC, but not as the predominant mechanism. Jones et al. (1995) also described that a decrease in hydrophobicity may contribute partially to the decrease in binding.

The influence of *Candida krusei* surface hydrophobicity on the adhesion to HeLa cells was compared to that of *Candida albicans* by Samaranayke et al. (1995). These authors found a positive correlation between cell surface hydrophobicity and adherence of *C. krusei* to HeLa cells but no such relationship was observed for *C. albicans*. Henriques (2005) also found no relationship between *Candida albicans* and *Candida dubliniensis* adhesion to HeLa cells and cell surface physico-chemical properties.

Kltotz (1994) studied the contribution of electrostatic forces to the adherence of *Candida albicans* to substrates and concluded that electrostatic forces although present in the process of adherence of yeast cells to some substrata, is a minor force which makes only a modest, at best, contribution to adherence.

ENVIRONMENTAL FACTORS AFFECTING ADHESION

Yeast Morphogenesis

Candida albicans undergoes reversible morphological transitions between ovoid, unicellular budding cells (yeast cells or blastospores) and chains of filamentous cells. The latter cell morphism displays different degrees of filamentation, ranging from slightly elongated ovoid cells to significantly extended tube-like cells. Filamentous cells are classified either as pseudohyphae or hyphae depending on their morphology. Although the degree of elongation of pseudohyphal cells can vary considerably, from relatively short to significant extended cells, they always display constrictions at their septa between individual cellular compartments. In contrast, true hyphae and their progenitors (germ tubes) show no constrictions, having parallel walls at their septa (Calderone 2002c).

Candida albicans ability to switch from yeast to hyphal growth in response to various environmental signals is directly related with its pathogenicity (Liu 2001). Prasad (1991) studied the contribution of dimorphic growth to virulence, determining the virulence of mutants that can grow only in either the yeast or filamentous form. Although virulence is decreased for such mutants, the strains used in those studies were produced by classical genetic methods and are likely to carry multiple genetic lesions.

There are evidences suggesting that yeast-hypha morphogenesis is co-regulated with other virulence factors. For instance, SAP4-6 genes, members of a large family of secreted aspartyl proteinase genes, that promote the virulence of *C. albicans* (Hube et al. 1997; Brown 2002), are expressed specifically during hyphal development. Of all the *Candida spp.*, only *C. albicans* and *C. dubliniensis* form both types of filamentous

growth (Calderone and Fonzi 2001), although, some species, as *C. glabrata*, can form pseudohyphae in response to nitrogen starvation (Csank and Haynes 2000).

Sugars

One of the main causes of oral Candidiasis is the presence of great amounts of carbohydrates in the oral cavity. Pizzo et al. (1999) studied the effect of some carbohydrates on the adhesion of some *Candida* species to epithelial cells. Their results indicate that incubation in sucrose or glucose significantly promotes adhesion of *Candida albicans* to epithelial cells. This is in agreement with previous studies by Samaranayke and MacFarlane (1982). They also found that diets rich in glucose or sucrose could influence the development and outcome of oral Candidiasis by enhancing *Candida albicans* adhesion, as well as *C. tropicalis* and *C. krusei*. The effect of glucose can be due to the production of a mannoprotein surface layer, which is known to enhance adhesion (McCourtie and Douglas 1985). Other studies (Samaranayake et al. 1986; McCullough et al. 1996) lead also to the conclusion that glucose can promote acid production and lower the pH, with consequent activation of acid proteinases and extracellular phospholipases, factors involved in yeasts adhesion. Other two important carbohydrates are fructose, which is present in fruits and honey and is used as a sucrose substitute in confectionery and maltose that is found in starchy foods accumulating on dental and prosthetic surfaces. These two carbohydrates, as well as glucose and sucrose, also enhance *Candida* adhesion to epithelium (Pizzo et al. 1999), although the extent of adhesion appeared to be lower in the presence of maltose. Besides the differences in the carbohydrates present in the growth media, adhesion is also dependent on the type of culture conditions (broth or solid media).

Temperature

Temperature of growth is known to affect cell morphology of dimorphic fungi including *C. albicans* (Ghannoum and Abu-Elteen 1991), thus affecting yeast cell-surface composition. The adhesion of *C. albicans* to BEC can be significantly modified by the temperature at which cells grow. Growth at 25°C increases the extent of adhesion when compared to cells grown at 37°C (Kennedy and Sandin 1988). According to Kennedy and Sandin (1988), *C. albicans* grown on Sabouraud Agar at 37°C was significantly more adhesive than when grown in Sabouraud broth. Growth phase of *C. albicans* has a marked influence on its adherence ability. Stationary phase yeasts, those cultured for periods longer than 18 h, were found to adhere to a greater degree than logarithmic-phase blastospores. However prolonged growth of the culture does not significantly enhance adherence (Calderone 2002b).

Inoculum Concentration

The inoculum concentration is determinant to the adhesion assays. There is no detectable yeast attachment at concentrations bellow 10^4 yeast ml^{-1}. In fact, 10^7 yeast ml^{-1} is the most used inoculum concentration. It has been reported that attachment of *C. albicans* to BEC/VEC (vaginal epithelial cells) gradually increases as the ratio of yeasts to epithelial cells in incubation mixtures is raised from 10:1 to 10000:1 (Kimura and Pearsall 1978; Lee et al. 1997). Kennedy and Sandin (1988) also reported that as the number of *Candida* that attached to epithelial cells increased, the percentage of BEC which had attached yeasts increased as well.

ASSESSMENT OF "IN VITRO" ADHESION

The study of *Candida* adhesion to epithelium involves three major steps, the preparation of the epithelial cells, the adhesion assay and the quantification of the adhesion extent. Table 1 presents a summary of the *Candida* species, epithelial cells origin, adhesion assays, and adhesion quantification methods used by different authors.

Adhesion Assays

Yeast adherence to epithelial cells varies considerably with the origin of the epithelium. The most used sources of epithelium are exfoliated samples like BEC. The method of BEC preparation was developed in 1978 by Kimura and Pearsall (1978) and is still being used today (McCarron et al. 2004) by most of the authors (see Table 1). Accordingly, BEC are obtained by gently rubbing the oral mucosa with sterile cotton swabs, followed by dispersion in sterile PBS. Cells are immediately used after centrifugation and washing with PBS.

The main problem is that BEC vary with the donor, the period of the day and other problems can arise, as a high number of non-viable cells, bacterial contamination and different degrees of enzymatic modifications of the cell surface (Cotter and Kavanagh 2000). These drawbacks were overcome by monolayer cultures. However, such cultures mimic neither the differentiation of cells during maturation, nor their interactions encountered *in situ*. A more realistic model seems to be reached with the combination of stromal equivalent and epithelial cells, commonly called organotypic cultures (Papaioannou 1998). Although this type of cells is convenient to use, invariably consists of heterogeneous mixtures of viable and non-viable cells and substantial cell-to-cell variation in the number of adhered yeast is always observed. Such preparations may also vary according to the donor, the time of sampling, the extent of colonization by the normal flora and the degree of exposure to various secretions. Therefore, it is more convenient to use a uniform cell population obtained by culturing epithelial cells. The most common cultured cells are HeLa (derived from cervical cancer cells taken from a woman named Henrietta Lacks) and KB (derived from an epidermal carcinoma of the mouth).

Recently, Schaller et al. (1998) used a model for reconstituted human oral epithelium (RHOE), maintained as multilayer natural cell cultures. It has been used as a satisfactory model for experimental Candidiasis (Jayatilake et al. 2005).

TABLE 1

Several parameters used in *Candida* adhesion to epithelium, including the source of epithelial cells, the *Candida* species adhered to that cells, the adhesion assay performed and the method used to quantify the adhered *Candida* and respective references

Source of epithelial cells	*Candida* species	Adhesion assay	Method for adhesion quantification	Ref.
BEC	*C. albicans*	Suspended coculture[1]	Scintilation	(Pla et al. 1996)
	C. albicans	Suspended coculture[1]	Safrannin staining	(Hazen 1989)
	C. albicans	Suspended coculture[1]	Gram staining	(Bailey et al. 1995)
	C. albicans	Suspended coculture[1]	Crystal violet staining	(Skoutelis et al. 1995)
	C. albicans	Suspended coculture[1]	Gram staining	(Sweet et al. 1995)
	C. albicans, *C. krusei*	Suspended coculture[1]	Gram staining	(Nair and Samaranayke 1996)
	C. albicans	Suspended coculture[1]	Crystal violet staining	(Jones and Gorman 1997)
	C. albicans	Suspended coculture[1]	Gram staining	(Ellepola and Samaranayke 1998)
	C. albicans	Suspended coculture[1]	Papanicolau stain	(Williams et al. 1999)
	C. albicans, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondi*	Suspended coculture[1]	Gram staining	(Ellepola et al. 1999)
	C. albicans	Suspended coculture[1]	Crystal violet staining	(Pizzo et al. 2001)
	C. parapsilosis	Suspended coculture[1]	Gram staining	(Panagoda et al. 2001)
	C. dubliniensis	Suspended coculture[1]	Direct observation	(Jabra-Rizk et al. 2001)
	C. albicans	Suspended coculture[1]	Crystal violet staining	(McCarron et al. 2004)
HeLa	*C. albicans*	24 well plates[2]	CFU determination	(Hazen 1989)
	C. krusei	24 well plates[2]	Gram staining	(Samaranayke et al. 1994)
	C. albicans, *C. tropicalis*, *C. krusei*	24 well plates[2]	Gram staining	(Pizzo et al. 1999)
	C. albicans	96 well plates[3]	CFU determination	(Bektic et al. 2001)
	C. albicans, *C. glabrata*	24 well plates[2]	Gram staining	(Dorocka-Bobkowska et al. 2003)
	C. albicans, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis*	24 well plates[2]	CFU determination	(Gruber et al. 2003)
HSC-3	*C. albicans*, *C. glabrata*	24 well plates[2]	Gram staining	(Dorocka-Bobkowska et al. 2003)
SCC4, SCC15, OKF6/TERT-2	*C. albicans*	6/12 well plates[3]	Tryptan blue	(Dongari-Bagtzoglou and Kashleva 2003)
KB (CCL-17)	*C. albicans*	Suspended coculture[1]	[3H]glucose	(Steele et al. 2001)
Human Epidermal keratinocytes	*C. albicans*	96 well plates[3]	Inverted microscope	(Ollert et al. 1993)
Epithelium from gingival tissues	*C. albicans*, *C. tropicalis*, *C. glabrata*	48 well plates[3]	ATP measurement	(Nikawa H. et al. 2002)
Gingival keratinocytes	*C. albicans*	6/12 well plates[3]	Tryptan blue staining	(Dongari-Bagtzoglou and Kashleva 2003)
Oral epithelial cells	*C. albicans*	Suspended coculture[1]	[3H]glucose	(Steele et al. 2001)

[1]Epithelium and yeast cells adhering in suspension.
[2]Epithelium adhered to glass immersed on the well plates.
[3]Epithelium adhered to the bottom well plates.

According to Jayatilake et al. (2005), the advantages of this model are: its multilayer structure closely resembling the oral epithelium, the ability to artificially reproduce the internal milieu of the oral cavity and its ready off-the-shelf use for time-limited experiments. Schaller et al. (1998) showed that, histologically, RHOE resembles the normal human oral epithelium and the pathological changes that accompany Candidal invasion are akin to human disease.

Quantification Methods

The attachment of *Candida albicans* to buccal cells from rats was first measured by Liljemark and Gibbons (1973). A few years later Kimura and Parsall (1978) proposed a method for the study of *Candida* adhesion to BEC. This assay included the mix of equal amounts of BEC cells and yeast cells and incubation for 1 h at 37°C. After adhesion, cells were filtered and the filters were stained and the number of *Candida* adhered per 100 BEC was enumerated. After several modifications, Kennedy (1990) proposed a standardization of that method, allowing uniformity of the results obtained by different researchers. Although this method is still being used (Table 1), Samaranayke and MacFarlane (1981) described a new method in 1981 for studying adhesion to epithelium that includes the adhesion of epithelial cells to the bottom of the wells of tissue culture well plates or to glass coupons (inserted on the wells) prior to the addition of microbial cells. In this method, mammalian cells must be adherent to the supporting surface.

Among the methods used to enumerate yeast cells adhered to epithelium are: the visual counting, using light, fluorescence, and electron scanning or transmission microscopy and the Coulter counting of the radiolabelled yeast. The visual method allows to follow the adhesion to individual epithelial cells but is a very time consuming technique. The radiolabelling method seems to offer an attractive alternative in some situations, although it should always be remembered that leachable isotopes can produce misleading results.

The quantification of the adhesion extent varies with the type of epithelial cells used and consequently with the adhesion assay performed (Table 1). When yeast cells adhere in suspended cocultures with epithelial cells usually a direct microscopic method is used and the percentage of epithelial cells with adhered *Candida* is determined. Both gram staining and CFU determination are applied to cells grown in well plates. In the first case cells are observed under the microscope and the number of *Candida* is determined per mm^2. More recently a method involving ATP measurement was proposed (Nikawa et al. 2002). The authors developed an in vitro assay technique to extract cellular and fungal ATP separately, allowing a quantitative evaluation of the adhesion of *Candida* to monolayers of epithelial cells.

CONCLUSIONS

The large range of host tissues that can be colonized and infected by *Candida* species suggests that these organisms possess a large number of adhesive surface factors, very few of which have actually been characterized or their role is not yet well understood. Another point worth noting is that adhesion assays have been performed with a limited number of strains of each species and it is already well established that adhesion is strain dependent. Therefore, in order to deepen the understanding of the adhesion phenomenon, the studies should be conducted with different clinical isolates of *Candida* species and using different types of epithelium.

The prevalence of non-*Candida albicans Candida* (NCAC) species is emerging today. In opposition to *Candida albicans*, only few yeast-cell interaction mechanisms were found and described for NCAC species. So, as adhesion is considered one of the most relevant *Candida* virulence factors, it is becoming increasingly more important to understand the mechanisms that are involved and their role in the adhesion process of each NCAC species.

Different methodologies have been used to perform adhesion assays and to quantify the extent of adhesion. Consequently, it is difficult to meaningfully compare the results reported in literature because different cells and culture conditions were used in each case. Therefore, it is of utmost importance to standardize the methodologies used to assess yeast-epithelium adhesion.

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